The role of macrophages in atherogenesis

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Monocyte-derived macrophages and foam cells play a central role in atherogenesis. In vitro studies suggest that macrophages exhibit many functions relevant to lesion initiation, progression, and regression. Intimal foam cells function within an incompletely understood and complex network of cytokines, growth factors, and other mediators that vary in spatial and temporal distribution during lesion formation. Macrophages may modify lipoproteins to form derivatives that then modulate lesion formation. These cells also participate in local lipid metabolism within the atherosclerotic lesion by sequestering, processing, and exporting lipids. Macrophages participate in chronic immune and inflammatory aspects of plaque formation by elaborating a vast array of mediators, and by processing and presenting antigens to T-lymphocytes. The macrophage can elaborate both stimulators and inhibitors of smooth muscle cell migration and proliferation as well as regulate the elaboration of many constituents of the vascular matrix. Macrophages also express many of the enzymes involved in degrading matrix constituents. These cells may thereby play a central role in the remodeling of the extracellular matrix during smooth muscle cell migration, neovascularization, and plaque rupture. When a plaque is ruptured, macrophage-derived proteins that modulate blood coagulation and fibrinolysis participate in local clotting and contribute to lesion evolution as well as the transition from the chronic to the acute stages of atherosclerosis. The in vivo environment of the macrophage and foam cell is extremely complex, with multiple stimuli acting concomitantly. The information derived from in vitro studies of macrophage functions has yielded hypotheses that can now be tested *in vivo* using increasingly powerful experimental strategies.

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Introduction

> 'If now ... we trace the development of the atheromatous condition a little farther back, we come — anteriorly to the period when the pultaceous matter is found in the seat of the atheroma — across a stage where nothing more is found than fatty degeneration in its ordinary form of granule cells...' [1].

For over a century, students of atherosclerosis have recognized the accumulation of foam cells (Virchow's granule cells in the above quotation) as a hallmark of atherosclerosis. The controversy over the origin of foam cells in atherosclerotic lesions yielded to the application of modern methodology for cell typing [2–6]. Although smooth muscle cells can also take up lipid, monocyte-derived macrophages constitute the major source of foam cells in all stages of human and experimental atherosclerosis. This review will consider how recent work has expanded our understanding of the multiple mechanisms by which mononuclear phagocytes modulate atherogenesis.

Recruitment of monocytes during atherogenesis

The recruitment and transformation of circuating monocytes into foam cells requires adhesion, transmigration, and accumulation of lipid, processes whose mechanisms and consequences are increasingly well understood. As early as the 1950s, Poole and Florey [7] recognized that

Abbreviations

apo—apolipoprotein; IL–interleukin; MCP—monocyte chemoattractant protein; M-CSF—macrophage-colony stimulating factor; NO—nitric oxide radical; VCAM—vascular cell adhesion molecule.

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monocyte adhesion to the luminal surface of the endothelium and penetration into the intima characterized the earliest phases of experimental atherogenesis in rabbits, a finding widely replicated in other species [8–11]. As atherosclerotic lesions progress, the expanded intima generally develops a rich network of newly formed microvessels [12]. These neovascular plexi tend to occur in regions rich in macrophages [13]. In the latter stages of lesion development, the luminal surface of these microvessels provides a large surface area for potential monocyte recruitment into the evolving lesion.

Recent work has provided much new information regarding the molecular mechanisms that underlie the 'patchy alteration in the endothelial surface' responsible for monocyte attachment postulated by Poole and Florey [7] nearly 4 decades ago. We now recognize a number of adhesion molecules expressed on the surface of endothelial cells in response to various stimuli that bind various classes of leukocytes [14]. These adhesion molecules fall into two general groups, members of the immunoglobulin gene superfamily and the selectins [15]. Members of the immunoglobulin G gene superfamily of potential relevance to atherogenesis include intercellular adhesion molecule-1, whose cognate ligands are the β_2 integrins such as leukocyte-function associated antigen-1 on the leukocyte surface. Vascular cell adhesion molecule (VCAM)-1 is another member of the immunoglobulin G superfamily expressed on various cell types, including vascular endothelium. VCAM-1 interacts with very late antigen-4, a β_1 integrin expressed particularly on the surface of mononuclear leukocytes. Thus, VCAM-1 exhibits selectivity for monocytes and lymphocytes, precisely the types of leukocytes that accumulate within developing atheroma. Endothelial cells also show inducible expression of members of the selectin family of adhesion molecules such as E-selectin (formerly known as endothelial leukocyte adhesion molecule-1) or P-selectin (formerly known as GMP140 or as PADGEM), which interact preferentially with granulocytes rather than mononuclear cells. L-selectin (leukocyte adhesion molecule-1) expressed on the surface of leukocytes may interact with an as yet undefined ligand on endothelial cells.

The case of VCAM-1 merits particular attention in relation to monocyte recruitment during atherogenesis. Endothelial cells over nascent fatty streaks in rabbits with diet-induced or genetically determined hypercholesterolemia express VCAM-1 focally [16]. Endothelium in lesion-prone areas of the rabbit aorta express VCAM-1 as early as 1 week after initiating a hypercholesterolemic diet, before intimal macrophages accumulate [17]. Although these data support a possible role for VCAM-1 in monocyte recruitment, other adhesion molecules may also contribute to this process. It is noteworthy that in advanced human atheroma, microvascular endothelial cells express VCAM-1, supporting the possibility that these neovascular channels provide a portal for entry of mononuclear cells into the established lesion [18].

Once the monocyte attaches to the luminal endothelial cells, be they macro- or microvascular, chemoattractant stimuli may promote their transendothelial migration and entry into the intima. The best studied candidate chemoattractant in this regard is monocyte chemoattractant protein (MCP)-1 [15,19]. This cytokine potently stimulates directed migration of monocytes *in vitro*. Vascular endothelial cells, smooth muscle cells, and leukocytes can inducibly express MCP-1 *in vitro* [20–25]. The regulation of MCP-1 in monocyte-derived cells is a matter of controversy [19]. Furthermore, in situ observations in human and experimental atherosclerotic lesions indicate expression of this chemoattractant molecule [26,27].

The mechanism of transendothelial penetration of the leukocytes *in vivo* remains a matter of speculation. Engagement of the cognate ligands of adhesion molecules may yield transmembrane signals which could activate the migratory machinery of the leukocyte. The precise links between hypercholesterolemia and other pro-atherogenic stimuli and the expression of monocyte adhesion molecules and chemoattractants by vascular cells *in vivo* remain incompletely defined. The possibility that oxidative modification of lipoproteins by vascular cells may initiate a cascade of early events is under investigation [22,28].

Foam cell formation and lipid metabolism

Having entered the arterial intima, many mononuclear phagocytes differentiate into foam cells, a specialized type of macrophage. We now recognize that the accumulation of cholesteryl esters by macrophages occurs by the uptake of modified lipoprotein particles via specific membrane receptors. It was long appreciated that LDL suppresses the expression of its classical receptor [also known as the apolipoprotein (apo) B,E receptor] efficiently enough to prevent the accumulation of high concentrations of cholesteryl esters found in foam cells.

Brown and Goldstein [29] demonstrated the existence of a receptor for modified LDL using acetyl LDL as the model ligand. Krieger's group [30–33] subsequently cloned scavenger receptors I and II, members of a new family of transmembrane proteins, expressed by macrophages and other cells. The scavenger receptors can mediate lipid loading as even cholesterol-replete cells continue to express them. Macrophages within human atheroma express such scavenger receptors [31]. Circulating monocytes do not express the scavenger receptor which can be induced upon differentiation into macrophages *in vitro* [34]. Interestingly, macrophage-colony stimulating factor (M-CSF) can augment [34–36] and γ-interferon can supress expression of the scavenger receptor [37]. *In vivo*, oxidative modification represents one plausible pathway for confering upon lipoprotein particles the ability to bind scavenger receptors, a property modeled *in vitro* by acetylation. Recent evidence by Sparrow *et al.* [38] suggests the existence of an additional receptor that binds highly oxidized LDL particles, and might provide an additional pathway for macrophage lipid accumulation. The molecular characterization of this putative 'oxidized LDL receptor' is incomplete at present.

The lesional macrophage not only accumulates lipid removed from the extracellular space but can process and possibly return lipids to the circulation [35,39]. It has been known for many years that mononuclear phagocytes can produce large quantities of apoE [29]. This apolipoprotein, an excellent ligand for the classical LDL receptor (the apoB,E receptor), may play a role in reverse cholesterol transport. Overexpression of apoE in transgenic mice can reduce diet-induced hypercholesterolemia [40]. Exogenous administration of apoE also inhibits lesion formation in hypercholesterolemic rabbits [41]. Genetic manipulation to inactivate apoE in mice render these usually atherosclerosis-resistant rodents susceptible to florid aortic foam cell lesion formation when fed lipid-enriched diets [42–44]. This enhanced atherogenesis accompanies a striking rise in 'remnant'-type lipoprotein particles in the blood of these apoE-deficient mice [43]. Factors that augment apoE expression in foam cells such as M-CSF may thus retard progression or promote regression of atherosclerosis [36,45]. ApoE may also modulate the immune response and aspects of tissue repair [46]. In additional apoE, macrophages can produce another apolipoprotein, serum amyloid A3, a component of certain HDL [47]. The content of serum amyloid A molecules derived from macrophages as well as liver may influence the metabolism of HDL particles.

Lesional macrophages can also express lipoxygenases, enzymes that may catalyze oxidative modification of intramural lipoproteins, furnishing products suitable as substrates for the scavenger uptake pathways and potentially capable of eliciting locally acting mediators [22,48,49]. However, oxidative modification of lipoproteins by cells may not require lipoxygenases [50]. Within human atheroma macrophages also produce lipoprotein lipase, an enzyme important in the remodeling of lipoprotein particles [51,52]. These recent observations broaden the traditional concept that this enzyme acts endovascularly at the luminal surface, and point to potential ongoing metabolism of lipoprotein constituents within the arterial wall itself during atherogenesis.

Activation and mediator production by macrophages

Mononuclear phagocytes, when appropriately stimulated, can produce many biologically active mediators that can influence virtually all aspects of atherogenesis [53]. A complete description of these mediators lies beyond the scope of this brief review. However, we shall illustrate the gamut of macrophage-derived mediators by highlighting selected classes grouped by function. Although many of these mediators may play pathogenic roles in atherosclerosis, some may exert protective functions during this process.

Regulators of smooth muscle proliferation

Smooth muscle cell migration and multiplication are likely to contribute to the formation of arterial hyperplastic diseases, including atherosclerosis. Macrophages provide one likely source of paracrine stimulators of smooth muscle cell proliferation. Macrophages can produce forms of platelet-derived growth factor [54,55], a family of mediators capable of stimulating smooth muscle proliferation *in vitro* and migration of smooth muscle cell *in vivo* [56]. Macrophages can also produce members of the epidermal growth factor family of smooth muscle mitogens. These include transforming growth factor-α [57] and a newly recognized heparin-binding epidermal growth factor-like molecule [58].

Macrophages can also produce basic fibroblast growth factor [59,60]. More recently we have found high levels of acidic fibroblast growth factor in association with macrophages in regions of neovascularization of advanced human atheroma [13]. In vitro studies [13] confirmed that macrophage-like cells (THP-1 cells exposed to phorbol esters for several weeks) could transcribe the gene for acidic fibroblast growth factor. Thus, the macrophage in lesions may produce both 'classical' forms of fibroblast growth factor, and may stimulate proliferation of endothelial cells as well as smooth muscle cells during different phases of atherogenesis.

Colony stimulating factors were originally described as factors that supported the survival, proliferation, or development, of various classes of bone-marrow-derived cells. M-CSF, a product of macrophages and other cells within atheromatous lesions [36,61], for example, is required for survival of mononuclear phagocytes, and renders them more responsive to mitogens such as interleukin (IL)-3 or granulocyte-macrophage-colony stimulating factor. Under some circumstances, smooth muscle cells can also express c-*fms*, the receptor for M-CSF [62–64]. Thus, in addition to altering a number of macrophage functions potentially relevant to atherosclerosis, M-CSF may modulate smooth muscle cell replication.

Interleukins such as IL-1 α and β, like the colony-stimulating factors, were initially thought to act on leukocyte targets exclusively. We now recognize cytokines such as IL-1 produced in large quantities by activated mononuclear phagocytes as candidates for signaling many proatherogenic functions of intrinsic vascular wall cells, including eliciting the production of endogenous growth factors that may amplify mitogenic effects [65–67].

Inhibitors of smooth muscle cell proliferation

Mononuclear phagocytes not only produce stimulators of smooth muscle cell proliferation, but can elaborate growth inhibitors as well. For example, macrophages can produce prostaglandins such as prostaglandin E_2. Such prostanoids can inhibit proliferation of vascular

cells by raising intracellular levels of cyclic AMP [65]. Mononuclear phagocytes express an inducible form of the nitric oxide synthase, an enzyme that produces the nitric oxide radical ($NO^{\cdot}$) [68,69]. In high concentrations, $NO^{\cdot}$ exerts microbicidal actions important in host defenses against infectious agents. In the context of atherogenesis, $NO^{\cdot}$ may inhibit smooth muscle proliferation by increasing intracellular levels of cyclic GMP [70].

Certain cytokines and growth factors produced by macrophages can also inhibit cell proliferation. For example, tumor necrosis factor and IL-1, although they can promote proliferation of smooth muscle cells, inhibit the replication of endothelial cells. Transforming growth factor β can also inhibit smooth muscle proliferation under many experimental conditions *in vitro* [71–75]. Interestingly, transforming growth factor potently stimulates synthesis of interstitial forms of collagen by smooth muscle cells [76,77]. Thus, although it may limit the division of smooth muscle cells, it can promote accumulation of extracellular matrix during evolution of the atheromatous plaque [76].

Matrix deposition and remodeling

Metabolism of the extracellular matrix has received surprisingly little attention in recent atherosclerosis research, although the matrix makes up the bulk of most complicated atherosclerotic lesions. Remodeling of the extracellular matrix doubtless contributes to lesion evolution by permitting migration of smooth muscle cells during intimal thickening and of endothelial cells during intralesional angiogenesis. Regional weakening in the integrity of the extracellular matrix in macrophage-rich regions seems to account for the transition from chronic to acute atherosclerosis in many cases of plaque rupture [78]. Macrophages produce in a regulatable fashion many of the enzymes considered important in remodeling of the vascular matrix. These enzymes represent a superfamily known as metalloproteinases because they depend upon a co-ordinated zinc atom for their catalytic activity. These enzymes share structural similarity, common catalytic mechanisms, and all exist as pro-enzymes or zymogens requiring limited proteolysis for activation.

Macrophages in atheroma can express forms of stromolysins, enzymes capable of activating the zymogens of the matrix metalloproteinase family [79]. Macrophages elaborate interstitial collagenase, important for degrading types I and III collagen, the most abundant forms of this matrix protein in atherosclerotic lesions [80,81]. Matrilysin also derives from mononuclear phagocytes [82]. These cells can also produce elastolytic activity required to hydrolyze elastin, another important protein of the vascular matrix [83–85].

Other matrix metalloproteinases produced by macrophages include gelatinases capable of further hydrolysis of collagen peptides released by the action of specific collagenases [86]. Certain gelatinases also can degrade the forms of collagen found in the basement membrane in the vascular intima. Cytokines can modulate monocyte/macrophage expression of matrix metalloproteinases both positively and negatively [87,88]. In addition to producing these hydrolytic enzymes, macrophages can elaborate tissue inhibitors of metalloproteinases, endogenous antagonists of these enzymes [86]. Thus, macrophages within the atheroma possess a broad and tightly regulated complement of enzymes that can remodel the matrix of the lesion, and might contribute to its focal weakening at sites prone to plaque rupture.

Macrophages as regulators of coagulation, thrombosis, and fibrinolysis

One of the consequences of plaque rupture is acute thrombosis. It is now recognized that control of hemostasis depends not only on soluble factors in the blood, but on regulators produced by cells of the arterial wall and by leukocytes. In this regard, mononuclear phagocytes in the atheroma can express a number of these key regulatory functions. Notably, lesional macrophages contain abundant tissue factor, an initiator of the so-called 'extrinsic' pathway of blood coagulation [89,90].

Macrophages can also produce an endogenous antagonist of plasminogen activator, plasminogen activator inhibitor-1 [91]. This protein can inhibit the endogenous urokinase or tissue-type plasminogen activators associated with macrophages and impede fibrinolysis, and thus stabilize any clot that may form in response to tissue factor. As in the case of several of the other regulatory systems described above, the macrophage has an entire complement of activities which can regulate local tendencies for blood clots to form and lyse [92].

Heterogenous functions of lesional macrophage populations

Our appreciation of the subtleties of macrophage functions has increased substantially in the past few years. In situ analysis of many of the specific functions of macrophages indicates considerable heterogeneity in their expression within the atherosclerotic lesion. That is, even within the same atheroma, different macrophages may express many specific functions to a varying degree. Indeed, such heterogeneity appears the rule rather than the exception during atherogenesis.

For example, replication of macrophages occurs much more commonly in advanced atherosclerotic lesions than had been previously appreciated [93,94]. Yet, only a fraction of the mononuclear phagocytes within a lesion divide at any given time in the life history of that lesion. Similarly, only subpopulations of lesional macrophages appear to express a variety of other functions during any given 'slice in time' of atheroma sampling [13,27, 52,90,95,96]. Some of this heterogeneity in expression

of specific macrophage functions may reflect the length of time in which a macrophage has resided within a lesion. The particular spectrum of macrophage foam-cell functions expressed by a given lesional cell may depend upon a local microenvironment of stimulatory and inhibitory cytokine signals. Alternatively, regional location of a macrophage may determine its functional state. For example, within the 'necrotic core' of advanced atherosclerotic plaques, necrobiosis of macrophages may occur. This cell death may result from accumulations of toxic concentrations of modified lipoprotein derivatives, of NO·, or local depletion of the 'survival factor' M-CSF.

It is apparent that lesional macrophages can express certain functions episodically in response to systemic stimuli. For example, cytokines can often induce their own expression and expression of other cytokines within mononuclear phagocytes. It is conceivable that systemic cytokinemia, such as might be encountered by a systemic viral or bacterial infection, could evoke an 'echo' in macrophages resident within the atheroma, eliciting a wave of local induced cytokine expression [97]. In this way, macrophages resident in atheroma could provide an internal amplification loop for propagation of pro-inflammatory mediators in response to a systemic stimulus [98].

Inconsistencies in the literature caution against a simplistic view of activation of macrophage functions by modified lipoproteins. Some studies find that modified lipoproteins augment cytokine production or other pro-inflammatory functions of macrophages [99–102], whereas others find the opposite [103]. These apparently paradoxical results from reliable laboratories likely reflect different types of stimuli (highly versus 'minimally' modified LDL) and disparate assay cell types (mouse peritoneal macrophages versus human monocyte-derived macrophages). Such confusion highlights the variability of our experimental preparations, and the substantial differences between most mononuclear phagocytes studied *in vitro* and lesional foam cells, which themselves show considerable disparity in function as discussed. While cell culture studies provide a foundation for hypothesis generation, ultimately in vivo observations will furnish data to resolve these seeming contradictions.

Conclusions

Macrophages play many roles during atherogenesis. Their functions intersect and influence diverse regulatory pathways including lipid metabolism, immune and inflammatory responses, growth control, matrix accumulation and remodeling, and thrombosis. We understand increasingly well the regulation of these various macrophage functions *in vitro*. Yet much of our extrapolation to atherogenesis in the intact animal, and in humans in particular, remains speculative. In the earlier stages of lesion initiation, increased adhesivity of monocytes to the endothelium by mechanisms currently understood in principle, provides a crucial initial step in the initiation of atherosclerosis. Formation of monocyte-derived foam cells is a hallmark of early atherogenesis. Sequestration of lipids within the macrophage may represent an appropriate host defense to an abnormal accumulation of potentially toxic components of native or modified lipoproteins in the vessel wall. The elaboration of apoE by lesional macrophages may promote reverse cholesterol transport, another potentially salutatory action of macrophages during atherogenesis. By the same token, macrophages can produce oxygen radicals that might promote modification of lipoproteins. Such modification modulates the ability of lipoprotein constituents to elicit pro-inflammatory cytokines, growth factors, or other functions. The 'pultaceous' debris described by Virchow [1] may arise from necrobiosis of macrophages overburdened with toxic lipids and deprived of essential survival factors.

There is increasing appreciation of the chronic immune and inflammatory aspects of atherosclerotic lesion formation [104]. Macrophages are classical antigen-presenting cells, capable of processing antigens and presenting nominal antigens to T lymphocytes, initiating the cellular immune response. The presence of chronically stimulated T cells within lesions [105], and the widespread expression of class II histocompatibility antigens by lesional macrophages indicates that macrophages can participate decisively in the local immune response that occurs during atherogenesis.

Because the macrophage can elaborate both stimulators and inhibitors of smooth muscle cell proliferation, these cells may also participate in the control of replication of smooth muscle and other cells during atherogenesis. Many of the same factors that modulate the proliferative response also critically regulate the biosynthetic pathways that lead to matrix accumulation during lesion formation. Smooth muscle migration and plaque rupture during the evolution and complication of atheroma depend on matrix breakdown as well as synthesis. As macrophages can express many of the enzymes involved in degrading matrix constituents, these cells may not only control the synthesis but also the breakdown of matrix materials. The regulation of matrix remodeling, both temporally and spatially, may prove pivotal in the natural history of an atheroma.

The macrophage can also express many components of the machinery that regulate blood compatibility as expressed above. Once a plaque is ruptured, the coagulation proteins in the blood gain access to macrophage-derived tissue factor which can initiate local clotting. The consequent acute arterial thrombosis can lead to the dramatic acute clinical manifestations of atherosclerotic disease, including acute myocardial infarction or stroke.

Finally, we must continue to increase our appreciation of the subtlety of the activation programs of macrophages. We will not understand atherogenesis fully until we unravel the complexities of regulation of the various specific functional characteristics of these multipotent cells. Although in vitro studies provide inspiration, we must exercise considerable caution when extrapolating studies on macrophages and cell lines in culture to the behavior of foam cells within atheroma. The complexity, range, and heterogeneity in space and in time of macrophage functions present a worthy challenge for future investigation.

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